4500
FS-SO-2203-1.40
(Problem 2)

DOES NITROGEN FIXATION OCCUR IN BARK BEETLES?

Prepared by:

J. R. Bridges — 7/20/81
Research Entomologist — Date

Approved by:

Peter L. Lorio, Jr. — 7/20/81
Project Leader — Date
RWU 2203

Introduction

Evidence for nitrogen fixation in insects has been found with the acetylene reduction assay for several species of termites (4, 6, 15, 28) and for a wood-eating cockroach (7). Citernesi et al. (11) suggested that nitrogen fixation occurs in the gastrointestinal tracts of two other insect species. Nitrogen-fixation in bark beetles has not yet been proven; but Peklo and Satave (26) showed an increase in nitrogen content of macerated Ips beetle larvae cultured in nitrogen-free medium.

On finding that southern pine beetle, Dendroctonus frontalis Zimm., and its microorganism complex cause an increase in the total nitrogen concentration of pine inner bark, Hodges et al. (16) suggested that bark beetle symbionts might be able to fix atmospheric nitrogen. So I conducted this study to find out whether nitrogen-fixing bacteria are associated with bark beetles and influence their nitrogen economy.

Materials and Methods

Beetles. I collected larvae of black turpentine beetles, Dendroctonus terebrans (BTB), from naturally infested loblolly pines, Pinus taeda L. Larvae were removed aseptically from standing trees or stumps or from bolts that had been taken to the laboratory. Emerging adult D. frontalis were collected from naturally infested loblolly pines with a refrigerated collection box similar to the one described by Browne (9). Ips avulsus and other life stages of D. frontalis were collected aseptically from bolts of naturally infested pines.

Isolation of nitrogen-fixing bacteria. The enrichment culture medium used to isolate nitrogen-fixing bacteria was (24): Solution I contained K_2HPO_4 (6.3 g); $NaH_2PO \cdot H_2O$ (1.9 g); $MgSO_4 \cdot 7H_2O$ (0.1 g); $Na_2MoO_4 \cdot 2H_2O$ (0.008 g); ferric citrate (0.008 g); sodium thioglycollate (0.5); yeast extract (0.2); resazurin (0.001); distilled, deionized H_2O (950 ml). Solution II was 50 ml of a 20% glucose solution. The two solutions were autoclaved separately before being mixed together. I added 15 g of Bacto agar to solution I to get a solid medium.

Single beetles, their frass,[1/] and samples of inner bark next to beetle galleries were separately crushed with a glass rod in Pankhurst tubes (10) containing 5 ml of medium, in 50-ml serum bottles containing 10 ml of medium, or in 125-ml serum bottles containing 40 ml of medium. Beetle macerates were flushed with sterile nitrogen, incubated at 26-28 C for 3-5 days, and then tested for acetylene reducing activity. At first, I cultured some beetles on an Azotobacter medium to check for aerobic nitrogen fixers (8), but none were present.

[1/] Debris or excrement produced by larvae.

Enrichments that were positive for acetylene reduction were subcultured in Pankhurst tubes or serum bottles and retested for acetylene reduction. Cultures from BTB that reduced acetylene through two subcultures were diluted serially and plated on the solid enrichment medium. After incubating the plates anaerobically in a GasPak jar (BBL), I picked individual colonies and isolated pure strains by successive streaking on the enrichment medium or on tryptic-soy agar (Difco).

Most probable number of nitrogen-fixing bacteria was estimated by the three-tube method (12). Five surface-sterilized D. terebrans, 15 I. avulsus larvae, or 0.05 g of inner bark tissue were homogenized in a glass tissue homogenizer and diluted with the enrichment medium. BTB larvae were surface-sterilized by Barras' (2) method before homogenization. The number of positive tubes was determined by the acetylene reduction assay.

Acetylene reduction assay. I assayed cultures for acetylene reduction by adding 1 ml of acetylene to each Pankhurst tube or serum bottle (27). After these had incubated for 24 h, I measured ethylene production with a Hewlett-Packard Model 5830A gas chromatograph equipped with a flame ionization detector. A glass column (I.D. 6 ft x 1/4 in) was packed with Porapak T (80/100 mesh, Waters Associates, Inc.). The carrier gas (N_2) flow rate was 40 ml/min, and the column temperature was 100°C.

To determine the rates of acetylene reduction by isolated bacteria, I inoculated sterile enrichment medium in 16 x 150 mm test tubes with 0.5 ml of a culture grown overnight in the medium containing 1.0 g/l of casamino acids. Test tubes were capped with screwcaps containing teflon-lined septa (Supelco) and gassed with sterile nitrogen for 10 min. After incubating at 28°C for 24 h, the cultures were flushed with Ar, and 1 ml of acetylene was added. The cultures were then shaken in a water bath at 28°C for 1 h. Acetylene reduction was stopped with 0.5 ml of 20% perchloric acid added to each tube. Protein was determined by the Folin method (22) on samples digested with 5 M NaOH at 80°C for 20 min. Bovine serum albumin was used as the protein standard.

For in situ acetylene reduction assays, about 10 BTB larvae (80-200 mg total live weight)--or 10 BTB adults, or 1.5 g of bark next to larval feeding areas--were placed in 9-ml screw-capped vials equipped with teflon-lined septa (Supelco). Larvae were assayed at all stages of development. For assaying BTB, I replaced air with Ar:O (80:20); for bark assays, I used Ar. One ml of acetylene was added to each vial, and the vials were incubated for up to 24 h at 26-28°C before gas samples were removed for ethylene analysis. Control vials without beetles or bark were used to correct for trace amounts of ethylene in the acetylene. Acetylene was purchased from Matheson Gas Products and contained approximately 0.007% ethylene. Vials without acetylene were used to determine whether ethylene was produced by beetles or bark in the absence of acetylene. Each experiment was replicated at least three times.

Characterization of the isolates. Biochemical tests were performed by methods described by MacFadden (23). Nitrate broth, Simmon's citrate agar, urea broth, phenol red broth base for sugar fermentation tests, malonate broth, and ONPG differentiation disks were from Difco. Moeller KCN and decarboxylase broth bases were from BBL. Capti-Seal^{R} reagent (Marion Scientific Corp.) was used for the oxidase test.

Results

Occurrence of nitrogen-fixing bacteria. Nitrogen-fixing bacteria were found by enrichment culture in all three species of bark beetles studied (Table 1). Of BTB larvae cultured, 79.5% had nitrogen-fixing bacteria. Seventeen of the BTB larvae that were negative for nitrogen-fixing bacteria were from a tree that seemed abnormal because the galleries were overwet and resin-soaked and held many dead larvae. If larvae from this tree are ignored, over 92% of the BTB larvae were positive for nitrogen-fixers. All samples of frass and phloem surrounding the BTB galleries had nitrogen-fixers.

Nitrogen-fixing bacteria were found in Ips avulsus larvae, pupae, frass, and gallery bark (Table 1).

The only stage of D. frontalis that had nitrogen-fixing bacteria was the adult emerging from infested bolts in the laboratory. Because no nitrogen-fixing bacteria were found in any D. frontalis adults, larvae, or eggs that had been aseptically removed from the tree, some emerging adults may have been contaminated with nitrogen-fixing bacteria after they emerged from the billet.

Most probable number of nitrogen-fixing bacteria. Most probable number of nitrogen-fixing bacteria was determined for D. terebrans larvae, I. avulsus larvae, and phloem next to galleries. Most probable numbers of nitrogen-fixers from three samples of BTB larvae were 3.7×10^4, 2.1×10^4, and $> 11 \times 10^4$ bacteria/larva. Because larvae were surface-sterilized, nitrogen-fixers must have been localized within the insect, probably in the gut. Five bark samples from BTB galleries had a mean of 2.2×10^5 bacteria/g of wet weight (SD = 4×10^5). Most probable numbers for a sample of 15 I. avulsus larvae showed fewer than one bacterium/larva. Three samples of phloem next to I. avulsus galleries had 0, 1.7×10^3, and 7.7×10^3 bacteria/g of wet weight.

Characteristics of nitrogen-fixing bacteria. Bacteria isolated from BTB larvae were subjected to 29 biochemical tests. All isolates fermented glucose. They were gram negative; catalase positive; nitrate, oxidase, indole, and urease negative; did not produce H_2S on KIA; and did not deaminate phenylalanine. The other tests had various reactions (Table 2). Isolates were divided into 13 groups (Table 2) based mainly on the arginine dihydrolase, lysine, and ornithine decarboxylase reactions, and the Voges-Proskauer test (1, 14, 24). Forty-four of the isolates were Enterobacter agglomerans; five were E. aerogenes. The other 45 were biochemically atypical of previously described species and were tentatively classified as Enterobacter spp. Nitrogen-fixing Enterobacter have been isolated from several habitats (1, 5, 20, 24, 29, 31) including E. agglomerans from termites (28).

The rate of acetylene reduction of six randomly selected isolates averaged 4.8 (range = 2-10) $nmol \cdot h^{-1} \cdot \mu g^{-1}$ protein or 50.6 (range = 10-121) $nmol \cdot h^{-1} \cdot 10^{-8}$ cells. These values are similar to those previously reported for other isolates of Enterobacteriaceae (24, 25, 28). Using the above rates of acetylene reduction by nitrogen-fixing isolates and using the most probable number of bacteria present in larvae and gallery bark, I calculated the potential acetylene reduction. For larvae the value would be 0.028 $nmol \cdot larva^{-1} \cdot h^{-1}$; for bark it would be 0.11 $nmol \cdot g^{-1} \cdot h^{-1}$. These levels are high enough to have been detected by in situ acetylene reduction assays. However, no acetylene reduction was observed for BTB larvae or bark in situ.

Discussion

A diverse microflora exists with bark beetles (3), and nitrogen-fixing bacteria can now be included among those microorganisms that can be isolated. Nitrogen-fixing bacteria are widespread in the forest (1, 13, 18, 21, 30), so they may only be chance contaminants of bark beetles, especially D. frontalis and Ips species. But the high occurrence of nitrogen-fixing bacteria with BTB, and the large population found in larvae and bark, suggest that this association, at least, is more than chance. That no in situ acetylene reduction was detected suggests that nitrogenase synthesis in these bacteria had been suppressed. Because combined nitrogen can inhibit nitrogenase synthesis (19), the high content of total nitrogen in pine inner bark (0.4%, 17) would probably inhibit nitrogen-fixation. Also, removing beetles and bark from the tree might have disrupted nitrogen fixation.

Because no *in situ* acetylene reduction could be demonstrated for BTB (or associated bark tissue) definite conclusions cannot be made about how nitrogen-fixing bacteria influence BTB's nitrogen economy. But the constant occurrence of large populations of nitrogen-fixing bacteria with BTB suggests a symbiotic relationship. BTB was the only one of the three bark beetle species studied that had many nitrogen-fixing bacteria, though all three occupy the same ecological niche and are sometimes found in the same tree. Under certain conditions (depending on weather, tree vigor, overcrowding, etc.), BTB may not have enough nitrogen. Then nitrogen-fixing bacteria could be important. Also, nitrogen-fixing bacteria could affect BTB development in ways not related to their nitrogen-fixing ability. Understanding the role of nitrogen-fixing bacteria in the biology of BTB awaits the development of techniques for rearing germ-free cultures of BTB. Development of germ-free beetles could then be compared to that of beetles that had been re-inoculated with nitrogen-fixing bacteria.

Acknowledgments

I thank Billy S. Carson, Veterans Adminstration Hospital, Pineville, Louisiana, for assistance in identifying some of the bacteria.

References

1. Aho, P. E., R. J. Seidler, H. J. Evans, and P. N. Raju: Distribution, enumeration, and identification of nitrogen-fixing bacteria associated with decay in living white fir trees. Phytopathology 64, 1413-1420 (1974)

2. Barras, S. J.: Improved White's solution for surface sterilization of pupae of Dendroctonus frontalis. J. Econ. Entomol. 65, 1504 (1972)

3. Barras, S. J., and T. J. Perry: Interrelationships among microorganisms, bark or ambrosia beetles, and woody host tissue: an annotated bibliography, 1965-1974. U.S. Dep. Agric. For. Serv. Gen. Tech. Report SO-10 (1975)

4. Benemann, J. R.: Nitrogen fixation in termites. Sci. 181, 164-165 (1973)

5. Bergersen, F. J., and E. H. Hipsley: The presence of N_2-fixing bacteria in the intestines of man and animals. J. Gen. Microbiol. 60, 61-65 (1970)

6. Breznak, J. A., W. J. Brill, J. W. Mertins, and H. C. Coppel: Nitrogen fixation in termites. Nature 244, 577-580 (1973)

7. Breznak, J. A., J. W. Mertins, and H. C. Coppel: Nitrogen fixation and methane production in wood-eating cockroach, Cryptocercus punctulatus Scudder (Orthoptera:Blattidae). Univ. Wis. For. Res. Notes 184 (1974)

8. Brown, M. E., S. K. Burlingham, and R. M. Jackson: Studies on Azotobacter species in soil. 1. Comparison of media and techniques for counting Azotobacter in soil. Plant Soil 17, 309-319 (1962)

9. Browne, L. E.: An emergence cage and refrigerated collector for wood-boring insects and their associates. J. Econ. Entomol. 65, 1449-1501 (1972)

10. Campbell, N. E. R., and H. J. Evans: Use of Pankhurst tubes to assay acetylene reduction by facultative and anaerobic nitrogen-fixing bacteria. Can. J. Microbiol. 15, 1342-1343 (1969)

11. Citernesi, U., R. Neglia, A. Seritti, et al.: Nitrogen fixation in the gastro-enteric cavity of soil animals. Soil Biol. Biochem. 9, 71-72 (1977)

12. Collins, C. H., and P. M. Lyne: Microbiological methods. 4th ed. Butterworths, London (1976)

13. Cornaby, B. W., and J. B. Waide: Nitrogen fixation in decaying chestnut logs. Plant Soil 39, 445-448 (1973)

14. Ewing, W. H., and M. A. Fife: Enterobacter agglomerans (Beijerinck) comb. nov. (the herbicola-lathyri bacteria). Int. J. Syst. Bacteriol. 22, 4-11 (1972)

15. French, J. R. J., G. L. Turner, and J. F. Bradbury: Nitrogen fixation by bacteria from the hindgut of termites. J. Gen. Microbiol. 95, 202-206 (1976)

16. Hodges, J. D., S. J. Barras, and J. K. Mauldin: Amino acids in inner bark of loblolly pine, as affected by the southern pine beetle and associated microorganisms. Can. J. Bot. 46, 1467-1472 (1968)

17. Hodges, J. D., and P. L. Lorio, Jr.: Carbohydrate and nitrogen fractions of the inner bark of loblolly pines under moisture stress. Can. J. Bot. 47, 1651-1657 (1969)

18. Jones, K.: Nitrogen fixation in the phyllosphere of the Douglas fir, Pseudotsuga douglasii. Ann. Bot. 34, 239-244 (1970)

19. Knowles, R.: The significance of asymbiotic dinitrogen fixation by bacteria. Treatise Dinitrogen Fixation 4, 33-83 (1977)

20. Koch, B. L., and J. Oya: Non-symbiotic nitrogen fixation in some Hawaiian pasture soils. Soil Biol. Biochem. 6, 363-367 (1974)

21. Larsen, M. J., M. F. Jurgensen, and A. E. Harvey: N_2 fixation associated with wood decayed by some common fungi in western Montana. Can. J. For. Res. 8, 341-345 (1978)

22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275 (1951)

23. MacFaddin, J. F.: Biochemical tests for identification of medical bacteria. Williams & Wilkins Co., Baltimore, Md. (1976)

24. Neilson, A. H., and L. Sparell: Acetylene reduction (nitrogen fixation) by Enterobacteriaceae isolated from paper mill process waters. Appl. Environ. Microbiol. 32, 197-205 (1976)

25. Papen, H., and D. Werner: N_2-fixation in Erwinia herbicola. Arch. Microbiol. 120, 25-30 (1979)

26. Peklo, J., and J. Satava: Fixation of free nitrogen by bark beetles. Nature 163, 336-337 (1949)

27. Postgate, J. R.: The acetylene reduction test for nitrogen fixation. In: Methods in Microbiology, Vol. 6B, edited by J. R. Norris and D. W. Ribbons, pp. 343-356. Academic Press, London (1972)

28. Potrikus, C. J., and J. A. Breznak: Nitrogen-fixing Enterobacter agglomerans isolated from guts of wood-eating termites. Appl. Environ. Microbiol. 33, 392-399 (1977)

29. Raju, P. N., H. J. Evans, and R. J. Seidler: An asymbiotic nitrogen-fixing bacterium from the root environment of corn. Proc. Nat. Acad. Sci. 69, 3474-3478 (1972)

30. Sharp, R. F.: Some nitrogen considerations of wood ecology and preservation. Can. J. Microbiol. 20, 321-328 (1974)

31. Werner, D., H. J. Evans, and R. J. Seidler: Facultatively anaerobic nitrogen-fixing bacteria from the marine environment. Can. J. Microbiol. 20, 59-64 (1974)

Table 1. Occurrence of nitrogen-fixing bacteria with bark beetles.

Insect	Number cultured	Positive C_2H_2 reduction
D. terebrans		
Larvae	122	97
Frass	6	6
Gallery bark	3	3
D. frontalis		
Emergent adults	36	8
Attacking adults	24	0
Callow adults	6	0
Larvae	87	0
Eggs	11	0
Gallery bark	30	0
Frass	26	0
I. avulsus		
Callow adult	13	0
Larvae	19	9
Pupae	10	1
Frass	2	2
Bark	2	2

Table 2. Identification and biochemical properties of nitrogen-fixing bacteria from D. terebrans.

Group	Classification	No. Isolates	Reaction[ab] A	L	O	VP	M	C	KCN	Mot	Gel	Mal	Gas	Lac	Suc	Man	I	Sor	Ara	Raf	Rha	Mel	Xyl
1	Enterobacter agglomerans, Biogroup 1	8	0	0	0	100	88	25	12	100	100	100	0	100	100	100	0	100	100	88	100	100	100
2	E. agglomerans, Biogroup 2	3	0	0	0	0	100	100	0	67	33	100	0	0	100	100	0	0	100	0	100	0	100
3	" " , Biogroup G2	18	0	0	0	0	94	89	33	94	50	100	100	22	100	100	44	17	100	22	100	11	94
4	" " , Biogroup G3	14	0	0	0	100	28	86	21	100	100	100	100	86	100	100	0	100	100	86	93	86	100
5	" " , Biogroup G3, mannitol neg.	1	-	-	-	+	+	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	+
6	Enterobacter aerogenes	5	0	100	100	100	0	100	100	100	80	0	80	0	60	100	80	100	80	0	40	20	60
7	Enterobacter sp.	3	0	100	100	0	100	100	67	33	0	100	33	100	100	100	100	100	100	100	100	100	100
8	" "	29	100	0	0	100	0	100	45	100	86	93	97	66	100	100	3	100	100	0	100	3	97
9	" "	9	100	0	0	0	89	100	56	100	56	44	100	78	100	100	11	89	100	33	100	0	100
10	" "	1	+	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	+	+	-	+
11	" "	1	-	+	-	-	+	+	-	+	+	+	+	-	+	+	+	-	+	+	+	-	+
12	" "	1	-	-	+	+	-	+	+	+	+	-	+	-	-	+	+	+	-	-	-	-	-
13	" "	1	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	+

[a]A = arginine dihydrolase; L, O = lysine and ornithine decarboxylase; VP, M = Voges-Proskauer and methyl red tests; C, Mal = citrate and malonate utilization; KCN = resistance to KCN inhibition; Mot = motility; Gel = gelatin liquefaction; Gas = Formation of gas from glucose; Lac, Suc, Man, I, Sor, Ara, Raf, Rha, Mel, and Xyl = fermentation of lactose, sucrose, mannitol, inositol, sorbitol, arabinose, raffinose, rhamnose, melibiose, and xylose.

[b]Numbers indicate percent positive reactions.